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Transcription termination of RNA polymerase I due to a T-rich element interacting with Reb1p

(*Saccharomyces cerevisiae*/ribosomal genes/polymerase pausing/release element)

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ABSTRACT **All transcription terminators for RNA polymerase I (pol I) that have been studied so far, ranging from yeast to humans, require a specific DNA binding protein to cause termination. In yeast, this terminator protein has been identified as Reb1p. We now show that, in addition to the binding site for Reb1p, the yeast pol I terminator also requires the presence of a T-rich region coding for the last 12 nucleotides of the transcript. Reb1p cooperates with this T-rich element, both to pause the polymerase and to effect release of the transcript. These findings have implications for the termination mechanism used by all three nuclear RNA polymerases, since all three are known to pause at this terminator.**

Although transcription termination is a potential regulatory step for gene expression in eukaryotes (reviewed in ref. 1), the mechanism whereby it operates is still poorly understood. Our approach to this problem has been to develop *in vitro* termination assays for RNA polymerase I (pol I), one of the three nuclear RNA polymerases, and to use the *in vitro* reactions to identify DNA and protein components needed to specify a functional termination site. Our confidence in this approach was recently stimulated by the finding that a pol I terminator from the yeast *Saccharomyces cerevisiae* is capable of serving as a pause site for all three nuclear RNA polymerases (2). Thus, we have reason to hope that mechanistic insights gained by studying the interaction of this yeast terminator with pol I will be applicable to the other nuclear RNA polymerases as well.

In common with other characterized terminators for pol I, the yeast terminator contains an essential protein binding site, which must be correctly oriented to cause termination (ref. 3; recently reviewed in ref. 4). The protein that binds to the yeast pol I terminator has been cloned and is called Reb1p (2, 5). In addition to Reb1p bound to its site, termination absolutely requires the presence of some additional 5′ flanking sequence (2, 3). In this study, we have performed a systematic mutagenesis of the pol I terminator to more clearly delineate its DNA components. The results show that a T-rich sequence, coding for the last 10–15 nucleotides of the terminated transcript, somehow cooperates with Reb1p to cause both pausing and release by mechanisms that are still unknown.

MATERIALS AND METHODS

Transcription Termination Assays. Transcription termination assay mixtures contained only template DNA, purified yeast pol I, and recombinant Reb1p. A 3′ extension on the template was used to initiate transcription in the absence of accessory transcription factors. Also, a magnetic bead on the downstream end of the template was used to separate transcripts still bound to the template from released transcripts. All of these manipulations have been previously described, as have methods for site-directed mutagenesis of the termination region and preparation of recombinant Reb1p (2).

RESULTS AND DISCUSSION

Characteristics of the Transcription Termination Reaction. The time course of a typical pol I termination reaction is shown in Fig. 1. At each time point, transcripts still bound to the template were separated from released transcripts and the two classes of transcript were analyzed separately. An autoradiograph of the reaction products is shown in Fig. 1*A* and a quantitative graph of the time course is shown in Fig. 1*B*. We presume that the bound transcripts represent transcripts that are paused at the terminator and that they are the precursor to both released and readthrough transcripts. The bound transcripts accumulate to a steady-state level, consistent with both of those assumptions.

Magnetic beads were attached to templates on the terminus downstream of transcription initiation so as not to interfere with the initiation process. This has the negative effect of making readthrough (nonterminated) transcripts heterogeneous in length, probably because steric interaction with the bead prevents pol I from elongating to the end of the template. This heterogeneity makes it difficult to accurately quantitate the amount of readthrough transcripts on beaded templates. To circumvent this problem in the rest of this report, we routinely run two parallel reactions, one with beaded templates and the other with regular, nonbeaded templates. Nonbeaded templates are used to accurately measure the ratio of total RNA 3′-end formation at the terminator versus the amount of readthrough transcription. Examples of reactions with nonbeaded templates are shown in Fig. 2. In a separate set of reactions, beaded templates were used to measure the fraction of transcripts, truncated at position −4, which were paused (bound) versus released (supernatant). Autoradiographs of measurements using beaded templates are shown in Figs. 1*A* and 3. From these measurements, we can calculate the percentage of total transcripts that are paused at position −4, at the end of a 30-min reaction, and use this as a measure of the activity of pausing signals in the Reb1p–terminator DNA complex. We can also calculate release efficiency (ratio of paused to released transcripts at the end of a 30-min reaction) as a measure of activity of release signals in the Reb1p–terminator DNA complex.

Analysis of Clustered Mutations. We have analyzed a series of clustered mutations scanning the terminator region upstream of the Reb1p binding site and measured the effect of these mutations on total RNA 3′-end formation, percent pausing, and release efficiency. Fig. 4*A* shows the sequence of the wild-type pol I terminator from position −36 to +23 with the 11-bp binding site for Reb1p underlined. Below the wild-type sequence is shown a series of mutant terminators in which blocks of 3–6 nucleotides at a time are changed. These block changes scan the entire −36 to +23 region except for the

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Abbreviation: pol I, RNA polymerase I.

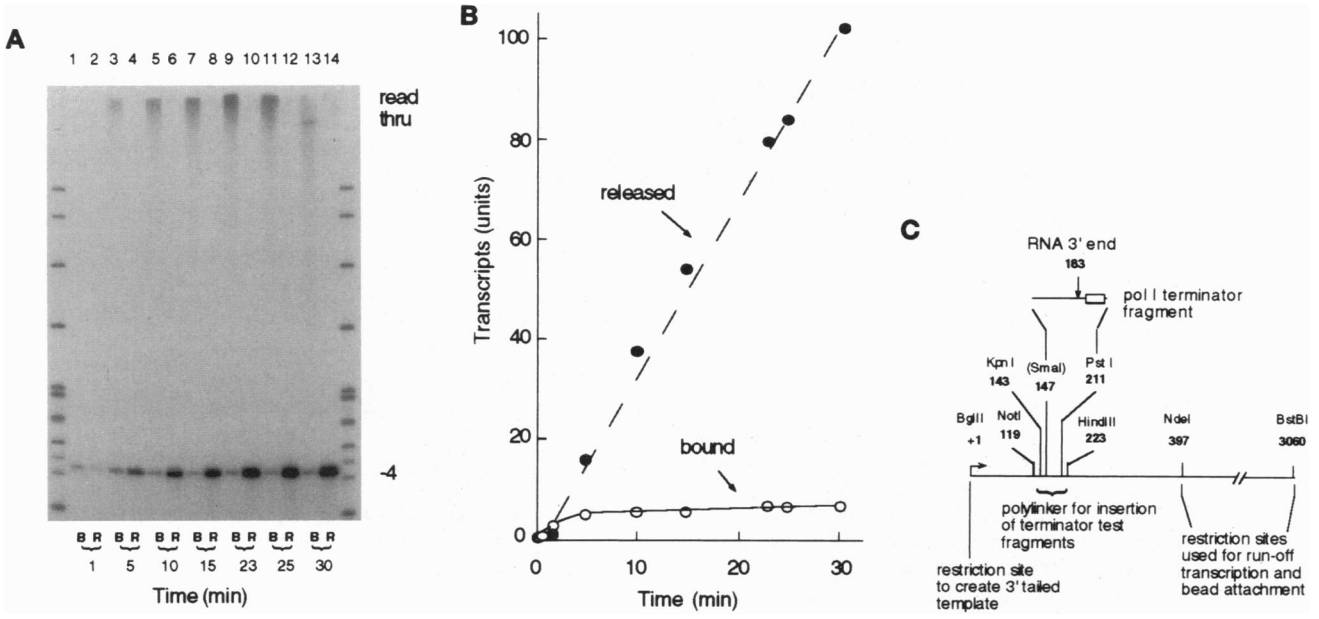

FIG. 1. Time course of transcript pausing and release during a pol I termination reaction. (*A*) Autoradiograph of transcripts separated by gel electrophoresis. The template in this reaction was mutant 51, which binds Reb1p tighter than does the wild-type template (see sequence in Fig. 4*A*). Templates were attached to magnetic beads so that at each time point transcripts could be separated into those bound to the template (B) and those that were released (R). Because of the bead, readthrough transcripts are somewhat diffuse and difficult to quantitate. Transcription of this template in the nonbeaded form (see Fig. 2, lane 4) shows that at 30 min only 7% of total transcripts have readthrough. (*B*) Graph of accumulation of bound (paused) and released transcripts. Autoradiograph shown in *A* was quantitated on a PhosphorImager to produce these data. If decay of the paused complex is first order, then (half-life of paused complex) = ln2(steady-state amount of paused complex)/(rate of synthesis of paused complex). We assume that all transcripts go through the paused state and then either release or become readthrough transcripts. This experiment shows that the total rate of RNA synthesis is linear over a 30-min reaction and the amount of paused complex reaches steady state in that time. Therefore, all the data needed to calculate half-life of paused complexes can be obtained from PhosphorImager analysis of gels, such as those shown in Figs. 2 and 3. Half-lives of complexes on lac repressor chimeric terminators were derived in a similar manner from unpublished data (S. W. Jeong, personal communication). (*C*) Diagram of template used for transcription termination assays. Numbers indicate distance in nucleotides from transcription initiation site. Small box indicates the 11-bp Reb1p binding site. Magnetic beads were attached at the *Bst*BI site. Nonbeaded templates were truncated at the *Nde* I site.

first 8 bp of the Reb1p binding site. We have previously shown that mutating any of these 8 bp eliminates both Reb1p binding as well as any detectable RNA 3′ end formation (3).

Fig. 2 shows an autoradiograph of an experiment in which all the templates listed in Fig. 4*A* were transcribed as nonbeaded templates, in the presence and absence of Reb1p, to measure their overall 3′-end-forming ability. The ability of each template to form RNA 3′ ends at position −4 is summarized graphically in Fig. 4*B*. In Fig. 3, each of the templates was attached to magnetic beads and the ratio of paused to released transcripts was measured. From these measurements, the percentage of total transcripts paused at position −4 was calculated for each template and the results of these calculations are summarized in Fig. 4*C*. From the data in Fig. 3, a

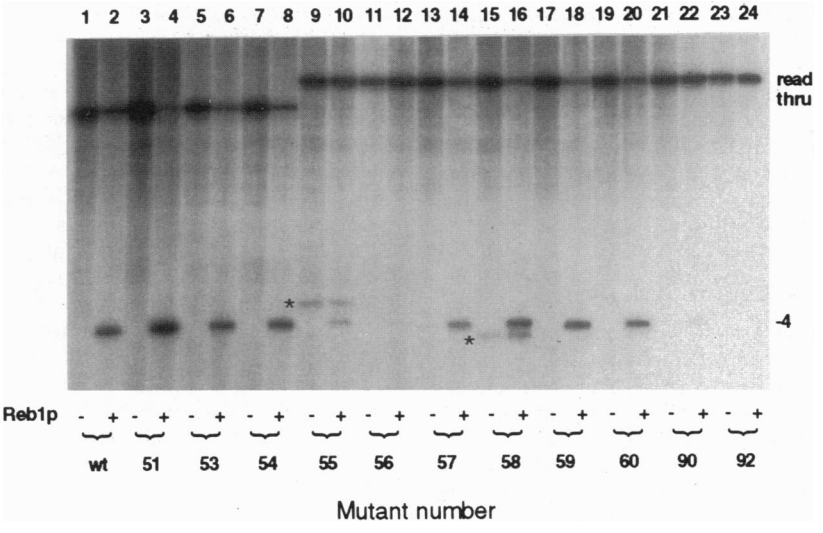

FIG. 2. Autoradiograph showing 3′-end formation at the pol I terminator versus readthrough, in the presence and absence of Reb1p, for each of the various wild-type and mutant terminators whose sequences are shown in Fig. 4*A*. Transcription proceeded for 30 min on nonbeaded templates. Asterisks denote RNA bands due to pausing induced by some feature of a particular G-block mutation. These transcripts are not dependent on Reb1p (shown in this figure) and they are not released (shown in Fig. 3).

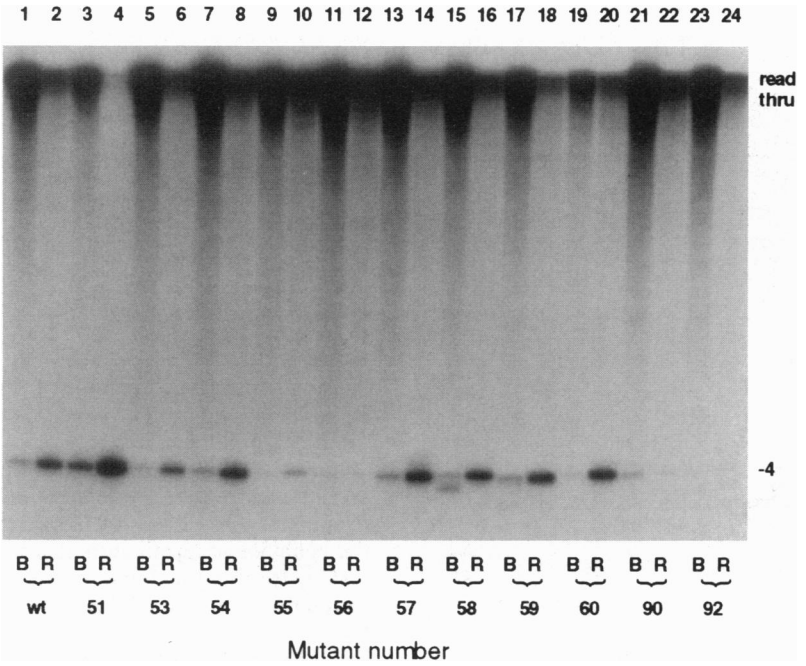

FIG. 3. Autoradiograph showing bound (paused) and released transcripts resulting from transcription of the various pol I terminators whose sequences are shown in Fig. 4A. Transcription proceeded for 30 min on beaded templates. Transcripts were separated into bound and released fractions prior to electrophoresis.

release efficiency was also calculated for each template, and these calculations are summarized in Fig. 4D. All of the calculations in Fig. 4 B–D are the averages of at least three independent measurements.

On a wild-type template, at the end of a 30-min reaction, ≈30% of total transcripts show 3′ ends mapping to position −4, while the rest have readthrough to the end of the template (Fig. 4B). On a wild-type template, 5–6% of total transcripts are still paused at −4 at the end of 30 min (Fig. 4C). Furthermore, 80–85% of transcripts with 3′ ends mapping to −4 have been released during this time (Fig. 4D). Several mutations we have tested do little or nothing to alter this wild-type behavior. For example, mutants 53, 54, 57, and 60 show pausing and release activities close to wild type. Mutants 58 and 59 consistently show pausing slightly above the wild-type amount (7–7.5% as opposed to 5–6%) but with completely normal release efficiencies. We conclude that the regions altered by these mutations play little or no role in the termination process.

In contrast, other mutations strongly alter termination and can be divided into two, or possibly three, classes. One class, of which mutant 51 is the only member, increases pausing but has no effect on the efficiency of transcript release (compare Fig. 4 B and C). Mutant 51 changes the last 3 nucleotides of the Reb1p binding site to Cs and creates a site with higher affinity for binding of Reb1p in filter binding assays (6). This correlation between binding affinity and percent pausing strongly supports the conclusion that Reb1p plays a major role in pausing.

A second class of mutations nearly eliminates pausing. These mutations (numbers 55, 56, 90, and 92) are all clustered over one region, which essentially codes for the last 12 nucleotides of the paused transcript (Fig. 4B). This region is very T-rich, and the two most severe mutations, numbers 92 and 56, alter a region containing only T residues interrupted by a single A. A major conclusion of this paper is that Reb1p-induced pausing of pol I requires this T-rich region. In the absence of either the T-rich region or Reb1, pausing is severely impaired.

It is difficult to decide whether or not the third class of mutants really constitutes a grouping distinct from the second class just described. This potential third class is represented by mutants 90 and 56, which are the only mutants of the entire collection that have a detectable effect on the efficiency of transcript release (Fig. 4C). It is clear that mutants that affect pausing overlap mutants that affect release and thus T-rich stretches are implicated in both processes. Comparison of Fig. 4 B and C indicates that mutating the upstream part of the T-rich region (number 90, for example) is more damaging to release, while mutating the downstream portion of the T-rich region (numbers 92 and 55) is more damaging to pausing. Further experimentation is necessary to determine whether this disparity is the result of one signal seen through two different lenses or whether it represents two distinct but overlapping signals.

Protein-Independent Pausing Caused by G Blocks. Examination of the autoradiograph in Fig. 2 shows that some G-block mutants induce transcript bands with 3′ ends mapping to positions other than −4. Since these minor bands occur in the absence of Reb1 (examples are bands marked by asterisks in Fig. 2, lanes 9 and 15), we think they are most likely due to Reb1p-independent pausing caused by the DNA structure of the G block itself. These extra bands are not released (Fig. 3, compare lane 15 with lane 16), presumably because pol I is not paused in a release permissive location. They are not due to GTP starvation since they still occur in the same amount when GTP levels are elevated (7). It will be of interest in future work to learn how a G block can cause pol I to pause.

Stimulation of Release As Well As Pausing by Reb1p. An additional conclusion drawn from these data is that Reb1p stimulates release of the transcript in addition to pausing the elongating polymerase. This conclusion comes from measuring the half-life of the paused complex when pausing is effected by Reb1p, as compared to the half-life when pausing is effected by a heterologous agent such as the lac repressor protein. We have previously made and tested chimeric pol I terminators in which the Reb1p binding site is removed and the binding site for lac repressor is substituted in its place (7). The lac repressor can be positioned so that it pauses pol I in precisely the same

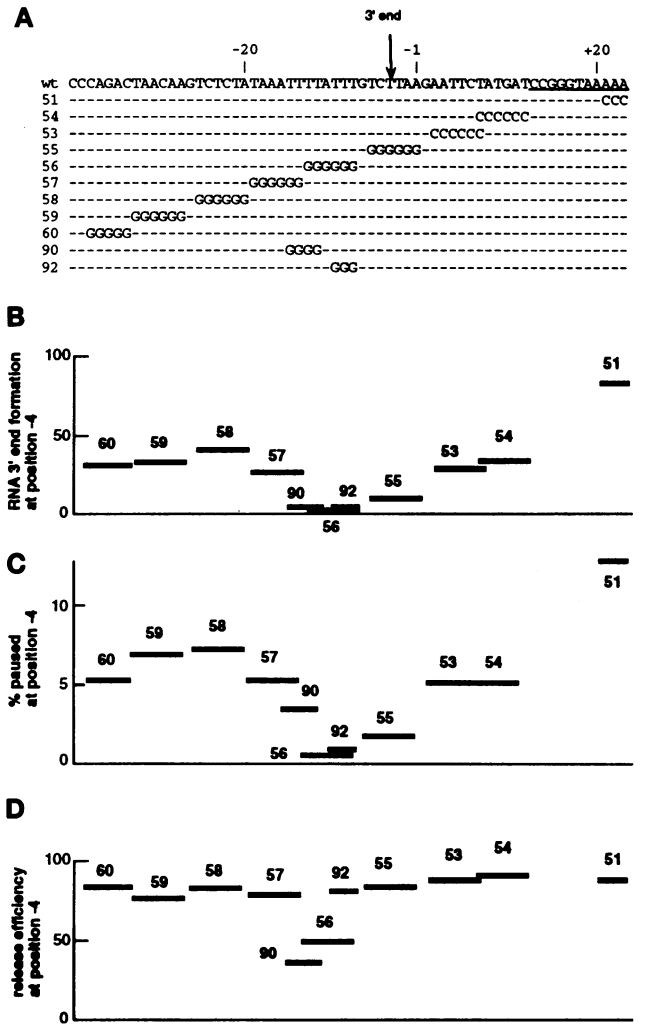

FIG. 4. Quantitative summary of the effect of block mutations on total RNA 3'-end formation, percent pausing, and release efficiency. (*A*) DNA sequence of wild-type pol I terminator from yeast (top line) plus various block mutants (lines 51–92). Sequence is numbered relative to a particular *Eco*RI restriction site. The 11-bp binding site for Reb1p is underlined and the 3' end of Reb1p-dependent paused or released transcripts maps to position −4. (*B*) Effect of block mutations on total RNA 3'-end formation at position −4. The intensity of bands at positions −4 and readthrough in Fig. 2 were measured with a PhosphorImager and were normalized for the length of the transcript. RNA 3'-end formation is expressed as the percentage of total transcripts with termini mapping to position −4. (*C*) Effect of block mutations on transcript pausing. Percent pausing is defined as molar fraction of total transcripts (paused, released, and readthrough) which, at the end of a 30-min reaction, remain bound to the template and have a 3' end mapping to position −4. (*D*) Effect of block mutations on transcript release. Release efficiency is defined as the fraction of transcripts with 3' ends mapping to position −4 which, at the end of a 30-min reaction, are not bound to the template. Note that transcripts paused at positions other than −4, including readthrough transcripts, are released very inefficiently even with the wild-type terminator.

position where Reb1p would pause it. In such chimeric terminators, >90% of all polymerases stop at the correct −4 position and eventually release their transcripts. However, release of paused transcripts is relatively slow (half-life of the paused complex is ≈9 min; S. W. Jeong, personal communication).

For comparison, mutant 51 of the yeast pol I terminator also causes the majority of transcripts to release at position −4 (83% in the experiment shown in Fig. 2, lane 4) but utilizes Reb1p to induce the pause. For mutant 51, however, the half-life of the paused complex is only ≈2.5 min, considerably shorter than the half-life when pausing is induced by lac repressor. Since in both types of terminator the paused complexes are primarily decaying by releasing the transcript, this shortened half-life suggests that Reb1p stimulates the rate of transcript release.

We have previously proposed a model in which pausing and release are two discrete steps in the overall termination process (2). The data in the current paper appear to support that model. However, we initially expected that pausing would be caused by Reb1p bound to its site and release would be effected by a separate element upstream of the Reb1p binding site. Contrary to that expectation, the data presented here show that Reb1p and the upstream T cluster cooperate in both pausing and release functions. Pausing and release appear to be concerted operations simultaneously affected by the same protein–DNA structures.

Summary of Pol I Terminator Structure. Fig. 5 summarizes our knowledge of the anatomy of the yeast pol I transcription terminator and points out an apparent discrepancy between the behavior of terminators that use Reb1p versus a chimeric terminator that uses lac repressor as the pausing agent. Line 1 shows the sequence of the wild-type Reb1p terminator, with the 3' end of the paused transcript indicated by an arrow and the 11-bp binding site for Reb1p surrounded by a box. Line 2 indicates regions that are involved in pausing in the Reb1p terminator, while line 3 indicates the region that affects transcript release. Line 4 summarizes the results of mutating a chimeric terminator using lac repressor as the pausing agent (7) and shows the region required for transcript release. This summary shows that the T-rich region required for polymerase pausing in the Reb1p terminator is essentially the same T-rich region that is required for transcript release in the chimeric lac repressor terminator. What is puzzling is that, in the Reb1p terminator, as long as there is any detectable pausing at all, the fraction of released transcripts is unaffected by most mutations of the T-rich region. Mutants 90 and 56 are an exception to this rule (see summary in Fig. 4*C*) but even they decrease release efficiency by only ≈50%. We might have expected, for example, to find mutants in which pausing occurred with normal efficiency but release was completely eliminated—but such is not the case.

To resolve this somewhat paradoxical result, we propose that, in terminators utilizing Reb1p, the same T-rich region is required for both pausing of elongating pol I as well as release of polymerase and transcript. Furthermore, Reb1p aids both

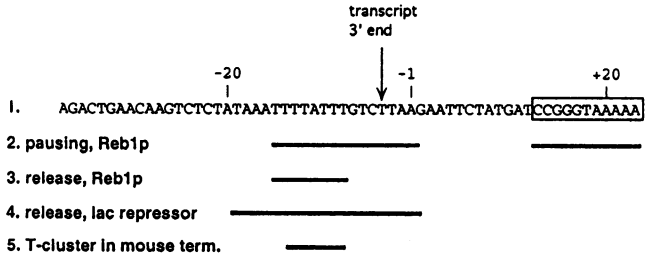

FIG. 5. Summary of DNA regions affecting transcription termination by pol I. Line 1, wild-type sequence of yeast pol I terminator with a box around the binding site for Reb1p. Line 2, bars indicate regions of the terminator required to pause the transcript (taken from Fig. 4*B*). Line 3, regions where mutation damages transcript release (taken from Fig. 4*C*). Line 4, summary of results from a previous study in which pol I was paused by lac repressor instead of Reb1p (7). Although release is slower in such a chimeric terminator than it is with Reb1p, the region needed for release also coincides with the T-rich element at the end of the paused transcript (indicated by a bar). Line 5, bar indicates location of a cluster of six Ts in the mouse pol I terminator, which have been implicated in transcript release (ref. 8; also see text).

processes. Reb1p is absolutely essential for pausing and is stimulatory for release. If, however, pausing is more sensitive to mutagenesis than is release, it will be difficult to obtain mutants in which only release is affected since we cannot study release in the absence of detectable pausing. Thus, the studies with the lac chimeric terminators were useful in clearly pointing out the release function of the T-rich element, a function that is more difficult to demonstrate when Reb1p is present.

Line 5 of Fig. 5 indicates the location of a T stretch in the mouse pol I terminator, which is located in the same relative position as the critical T-rich element of the yeast pol I terminator. Reinterpretation of previous work (7, 9) strongly suggests that this T stretch in the mouse terminator is essential for transcript release and at least partially dispensable for pausing. A similar T cluster is located in this approximate location in the pol I terminators from many species but there are notable exceptions, particularly among human pol I terminators (discussed in ref. 7). Thus, we are left with the conclusion that it is a T-rich element in many pol I terminators that cooperates with the appropriate DNA binding protein to effect termination. But the fact that some terminators do not have obvious T-rich elements indicates that there may be alternative solutions or else the signal provided by the T-rich element is more complex than at first appears.

The mutagenesis reported here appears to rule out the involvement of hairpin structures in pol I terminator function. However, in some ways the T cluster in the pol I terminator is reminiscent of T clusters, which are similarly located in many, but not all, intrinsic terminators of prokaryotes (8). Whether or not the T-rich regions in eukaryotic and prokaryotic terminators perform similar functions is an intriguing question for future research.

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